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CELL BIOLOGY AND METABOLISM:

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# S100A13 Is Involved in the Regulation of Fibroblast Growth Factor-1 and p40 Synaptotagmin-1 Release *in Vitro**

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**We have previously characterized the release of the signal peptide sequence-less fibroblast growth factor (FGF) prototype, FGF-1, *in vitro* as a stress-induced pathway in which FGF-1 is released as a latent homodimer with the p40 extravesicular domain of p65 synaptotagmin (Syn)-1. To determine the biologic relevance of the FGF-1 release pathway *in vivo,* we sought to resolve and characterize from ovine brain a purified fraction that contained both FGF-1 and p40 Syn-1 and report that the brain-derived FGF-1:p40 Syn-1 aggregate is associated with the calcium-binding protein, S100A13. Since S100A13 binds the anti-inflammatory compound amlexanox and FGF-1 is involved in inflammation, we examined the effects of amlexanox on the release of FGF-1 and p40 Syn-1 in response to stress *in vitro*. We report that while amlexanox was able to repress the heat shock-induced release of FGF-1 and p40 Syn-1 in a concentration-dependent manner, it had no effect on the constitutive release of p40 Syn-1 from p40 Syn-1 NIH 3T3 cell transfectants. These data suggest the following: (i) FGF-1 is associated with Syn-1 and S100A13 *in vivo*; (ii) S100A13 may be involved in the regulation of FGF-1 and p40 Syn-1 release in response to temperature stress *in vitro*; and (iii) the FGF-1 release pathway may be accessible to pharmacologic regulation.**

The FGF[1] prototype, FGF-1, functions as an extracellular mitogen for a diverse population of target cells, yet it lacks a classical signal peptide sequence for secretion (1). FGF-1 is released in response to temperature stress *in vitro* (2) as a latent FGF-1 Cys-30 homodimer (3, 4) through a secretion pathway that is independent of the conventional route mediated by the endoplasmic reticulum (ER)-Golgi apparatus (3) but is sensitive to inhibition by actinomycin D and cycloheximide (2).

We have partially characterized the latent FGF-1 species released *in vitro* in response to temperature stress. The extracellular FGF-1 homodimer is a component of a high molecular weight aggregate that contains the extravesicular p40 domain of synaptotagmin (Syn)-1 (5). The full-length Syn-1 translation product (p65) has been implicated in the regulation of exocytotic (6) and endocytotic (7) traffic and, like FGF-1 (4), Syn-1 is a phosphatidylserine- (8) and heparin-binding protein (9). Whereas the stress-induced FGF-1 and p40 Syn-1-containing extracellular aggregate is not biologically active and binds poorly to immobilized heparin (5), the biological and heparin-binding properties of FGF-1 can be recovered if the aggregate is treated with a reducing agent such as reduced glutathione (3) or with ammonium sulfate (2). It is possible that the latent character of this FGF-1 aggregate may represent a physiologic safeguard to ensure that extracellular FGF-1 can only signal if the extracellular environment is appropriate for the cell to respond to its stimuli; otherwise, it would be rapidly cleared. Indeed, cell-surface-associated heparin-sulfate proteoglycans are thought to not only present FGF-1 to its high affinity receptor tyrosine kinase (10) but also to protect FGF-1 from degradation by proteolytic enzymes present in the extracellular milieu during cell migration and proliferation (11, 12).

Because FGF-1 is released in response to heat shock *in vitro* as a reducing agent and denaturant-sensitive aggregate with p40 Syn-1 (5), and Syn-1 is required for FGF-1 secretion in response to heat shock (13), we sought to determine whether FGF-1 and Syn-1 also exist as an aggregate *in vivo* in order to provide a physiologic correlate to the *in vitro* data. Since neural tissue has served as the traditional source of native FGF-1 (14) and early evidence suggested that FGF-1 is present in neural tissue as an acid-sensitive high molecular weight aggregate (15), we sought to determine whether FGF-1 and p40 Syn-1 could be resolved as a heparin-binding aggregate from neutral extracts of neural tissue. We report that brain-derived FGF-1 exists as a multiprotein aggregate with p40 Syn-1 and S100A13, a member of the S100 gene family of calcium-binding proteins (16, 17) and have been able to utilize a pharmacologic strategy to illustrate that S100A13 may be a functional component of the FGF-1 release pathway.

## EXPERIMENTAL PROCEDURES

*Materials and Immunoblot Analysis*—Amlexanox (also known as AA673, Amoxanox, and Solfa) and its three derivatives were a very generous gift of Dr. G. Goto from Takeda Chemical Industries, Osaka, Japan. All other chemicals were reagent-grade and obtained from Sigma except where otherwise indicated. Chromatography solvents were HPLC-grade and obtained from Burdick and Jackson (Muskegon,

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[1] The abbreviations used are: FGF, fibroblast growth factor; Gal, galactosidase; RP-HPLC, reversed phase-high pressure liquid chromatography; Syn, synaptotagmin; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; IL, interleukin.

MI). The rabbit polyclonal antibodies against recombinant human FGF-1 and rat p40 Syn-1 were prepared as described previously (5, 13). All antibodies were used for immunoblot analysis at a concentration of 4 $\mu$g/ml in blocking buffer as described previously (2) except that an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech) was used for protein detection.

*Preparation of Brain Extracts*—Ten (1.5 kg) unstripped ovine brains (Pel-Freeze® Biologicals, Rogers, AR) were homogenized in 1.3 volumes of 50 mM Tris-HCl, pH 7.4, for 2 min in a Waring blender. The homogenate was centrifuged at 10,000 $\times$ *g* for 1 h, and the supernatant was filtered through sterile gauze. The filtrate was subjected to stepwise salt fractionation with 50 and 95% $(NH_4)_2SO_4$ saturation, and the precipitates were collected by centrifugation as described (14). The 95% $(NH_4)_2SO_4$ saturation precipitate was resuspended in 100 ml of 50 mM Tris-HCl, pH 7.4, and dialyzed for 18 h against 50 volumes of the resuspension buffer using a Spectra/Por ($M_r$ 12–14,000) dialysis membrane (Spectrum Medical Industries Inc., Houston, TX). All purification procedures were performed at 4 °C.

*Chromatographic Analysis of Neutral Brain Extracts*—A 2.5 $\times$ 22-cm plastic column containing 25 ml of hydrated heparin-Sepharose CL-6B was equilibrated with 10 volumes of 50 mM Tris-HCl, pH 7.4, and the brain extract was adsorbed twice over the immobilized heparin. The column was washed with at least 10 bed volumes of the resuspension buffer until the absorbance of the eluate at $\lambda$ = 280 nm was less than 0.01. Three batch fractions were eluted with 100 ml of 50 mM Tris-HCl, pH 7.4, containing 0.4 M NaCl, 0.7 M NaCl, and 1.5 M NaCl, and samples from each NaCl eluate (25 ml) were adsorbed to a C4 column (Vydac™, Hesperia, CA) conditioned in 0.1% trifluoroacetic acid (Pierce). RP-HPLC was performed as described (18) using a linear gradient of acetonitrile (40 to 100%) in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min, and the effluent was monitored at $\lambda$ = 214 nm. Samples were collected as absorbance peaks independent of volume in 1.0 M Tris-HCl, pH 7.4, in an attempt to maintain aggregate integrity and analyzed by FGF-1 and Syn-1 immunoblot analysis as described (2, 5) except that the ECL system was used for protein detection. Although many peaks exhibited the presence of both FGF-1 and Syn-1 by immunoblot analysis, only the 1.5 M NaCl heparin-Sepharose elution fraction contained a unique absorbance peak that contained both FGF-1 and Syn-1 by immunoblot analysis at a dilution of 1:100. This peak was re-chromatographed on a microbore 300-Å C4 Aquapore RP300 column (Perkin-Elmer), and bound proteins were eluted as absorbance peaks at $\lambda$ = 214 with a linear gradient (40–100%) of 70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid and a flow rate of 0.2 ml/min.

*Protein Sequencing*—Approximately 10 $\mu$g of protein from the indicated peaks in Fig. 1, *B* and *C*, were subjected to proteolytic digestion using lysyl endopeptidase C (Boehringer Mannheim) as described (73). Peptides were isolated by RP-HPLC using an Applied Biosystems model 130 separation system. Isolated peptides were subjected to automated Edman degradation using either an Applied Biosystems model 473A or 477A protein sequenator. Proteins were identified by comparison of the amino acid sequences obtained for several of these peptides against an NCBI (National Center for Biotechnology Information) protein sequence data base using the BLAST (Basic Local Alignment Search Tool) program.

*Pharmacology of FGF-1 Release in Response to Heat Shock*—NIH 3T3 cell FGF-1 [2] transfectants and FGF-1:$\beta$-gal and p65 Syn-1 cotransfectants (13) were grown to 80% confluence on fibronectin-coated dishes and subjected to temperature stress (41.5 °C, 90 min) under the conditions previously described (74). Where indicated, various concentrations of either amlexanox or one of its chemical derivatives were present during the entire heat shock period. A stock solution of amlexanox was freshly prepared in equimolar NaOH, and serial dilutions in phosphate-buffered saline were prepared prior to addition to the cells as recommended by Takeda chemical Industries Ltd. The compounds were added in volumes of 160 $\mu$l/20 ml of medium. The same volume of NaOH without drug was also added to the cells and found to have no effect on either protein release, membrane permeability (lactate dehydrogenase activity), or cell viability. In addition, cells maintained at 37 °C in the presence or absence of amlexanox for the same period were used as a control. Conditioned media were collected and filtered through 0.2-$\mu$m cellulose acetate. Aliquots (300 $\mu$l) were taken for analysis of lactate dehydrogenase enzymatic activity in the conditioned media according to an adaptation of the original method of Bergmeyer (75) and Sigma procedure DG1340-UV. The remaining filtrate was treated with fresh 0.1% (w/v) dithiothreitol for 2 h at 37 °C and processed by heparin-Sepharose chromatography as described (2).

## RESULTS

*Brain-derived FGF-1 Exists as a Denaturant-sensitive Aggregate with Syn-1*—Extracts of ovine brain were prepared at a neutral pH and subjected to $(NH_4)_2SO_4$ precipitation (50 and 95% saturation) as described under "Experimental Procedures." The 95% $(NH_4)_2SO_4$ saturation precipitate was resuspended as described previously (18) and separated into three batch-eluted fractions (0.4, 0.7, and 1.5 M NaCl) by heparin-Sepharose chromatography. Whereas immunoblot analysis of each eluate revealed the presence of both FGF-1 and p40 Syn-1 in all three fractions, only the 1.5 M NaCl post-heparin-Sepharose eluate was able to produce a reversed phase (RP)-HPLC peak (Fig. 1A) that contained both FGF-1 and p40 Syn-1 by immunoblot analysis (data not shown). The presence of p40 Syn-1 in the 1.5 M NaCl post-heparin-Sepharose elution fraction was unexpected since recombinant p40 Syn-1 has been observed to elute at a lower (~0.6 M NaCl) ionic strength [5]. To ensure that the post-RP-HPLC heparin-binding fraction containing both FGF-1 and p40 Syn-1 did not contain additional non-associated proteins, this fraction was again subjected to analysis by RP-HPLC. As shown in Fig. 1*B*, RP-HPLC analysis revealed a single symmetrical peak with a retention time identical to that previously observed (Fig. 1*A*). Immunoblot analysis using FGF-1 and Syn-1 antibodies confirmed the presence of both FGF-1 and p40 Syn-1 in this sample (Fig. 2, *A* and *B*).

Since this fraction (Fig. 1*B*) contained both FGF-1 and p40 Syn-1 and the electrophoretic mobility of the FGF-1:Syn-1 aggregate released in response to temperature stress is denaturant-sensitive (5), we anticipated that treatment of this fraction with a chaotropic agent should resolve the FGF-1 and Syn-1 components as individual peaks with RP-HPLC retention times identical to their retention times defined by both the native (Fig. 1*A*) and recombinant (Fig. 1*D*) proteins. Therefore, the brain-derived, post-RP-HPLC fraction (Fig. 1*B*) containing both FGF-1 and p40 Syn-1 was heated (5 min, 95 °C) in the presence of 8.0 M guanidine HCl and analyzed by RP-HPLC. As shown in Fig. 1*C*, the fraction containing both FGF-1 and p40 Syn-1 was present, but its absorbance was reduced significantly. In addition, numerous fractions with distinct retention times were readily visible including a major absorption peak and fractions previously defined as FGF-1 and p40 Syn-1 (Fig. 1, *A* and *D*). These data suggest that thermal and guanidine HCl denaturation of the brain-derived post-RP-HPLC fraction (Fig. 1*B*) containing both FGF-1 and p40 Syn-1 generates additional fractions with different retention times including peaks with retention times identical to FGF-1 and p40 Syn-1. Thus it is likely that this brain-derived, heparin-binding fraction represents an aggregate that contains FGF-1 and p40 Syn-1 as well as other temperature- and chaotropic-sensitive components.

*The S100 Gene Family Member, S100A13, Is a Component of the Brain-derived FGF-1 and p40 Syn-1 Aggregate*—Since the major absorption fraction containing both FGF-1 and p40 Syn-1 (Fig. 1*B*) demonstrated an unknown retention time, we sought to determine its structure. However, automated Edman degradation of this peak failed to yield any information. Therefore, this fraction (Fig. 1*B*) was subjected to LysC digestion, and the peptides were resolved by RP-HPLC. Automated Edman degradation of these peptides demonstrated that this fraction contained a member of the S100 gene family (17), the ovine homolog of human S100A13 (16). Interestingly, the structure of the S100A13 protein predicted from the bovine (GenBank™ accession number AB001567), murine, and human cDNA sequences (16) suggests that S100A13 contains 98 amino acid residues, 9 of which have cyclic side chains that absorb in the far-UV area used for detection (19, 20). Thus, the high UV

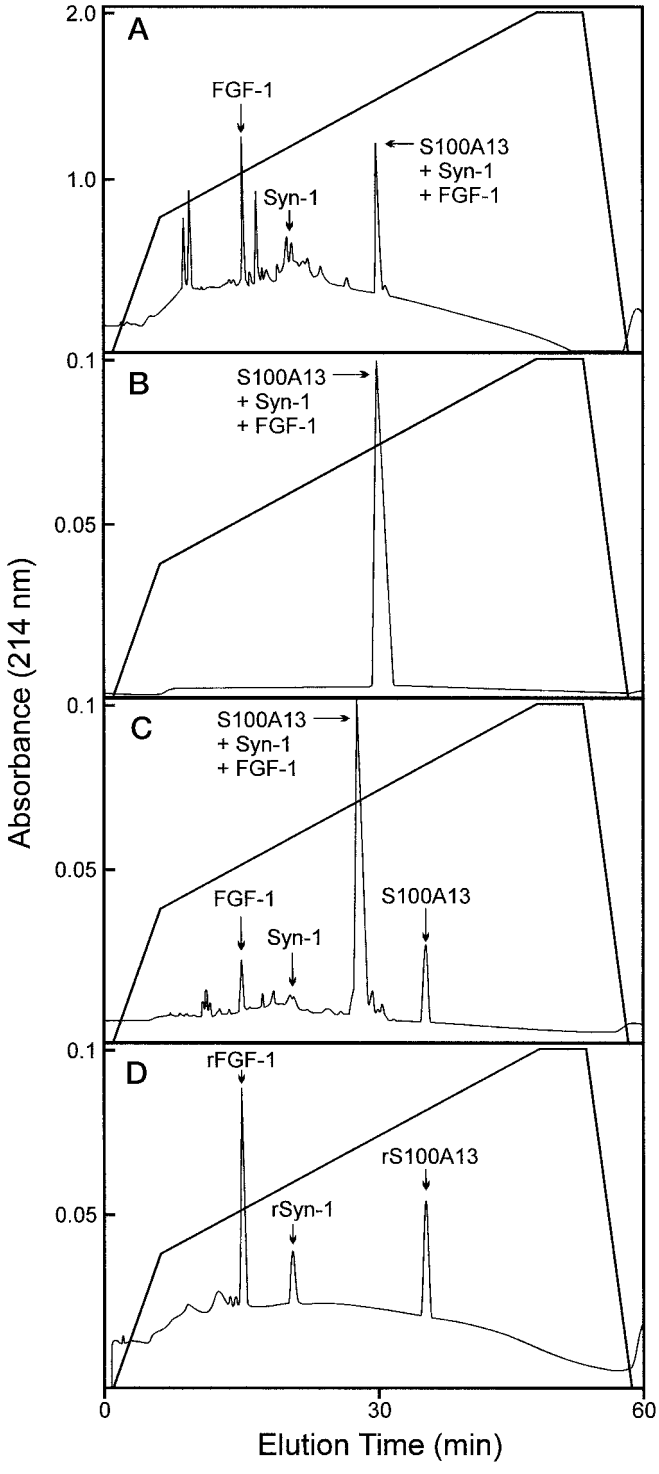


FIG. 1. **Purification of a heteromeric aggregate containing FGF-1, Syn-1, and S100A13 from ovine brain.** Ovine brain extract was prepared and separated into three fractions by heparin-Sepharose chromatography as described under "Experimental Procedures." Data are reported as a function of relative absorbance and retention time. The *solid line* on the chromatogram indicates the development of the acetonitrile gradient. *A,* RP-HPLC analysis of 25 ml of the post-heparin-Sepharose 1.5 M NaCl eluate. Fractions were collected as absorbance peaks into equal volumes of 1 M Tris-HCl, pH 7.4, to conserve aggregate integrity. Aliquots were taken along the chromatogram and probed for the presence of FGF-1 and Syn-1 by immunoblot analysis (data not shown). Although the majority of these fractions contained either FGF-1, Syn-1 or both, the fractions identified as FGF-1 and Syn-1 were determined by their presence as FGF-1 and Syn-1 immunoblot-positive samples at a dilution of 1:100. The presence of S100A13 was confirmed by microsequencing. *B,* the peak containing FGF-1, Syn-1, and S100A13 from *A* was rechromatographed by RP-HPLC to confirm its retention time and resolve it as a single peak. *C,* the fraction obtained after two consecutive runs containing FGF-1, Syn-1, and S100A13 was treated with of 8 M guanidine HCl in 0.25 M Tris-HCl, pH 8.5, vortexed for 1 min, heated for 5 min at 95 °C, vortexed for 1 min, and allowed to cool to room temperature before separation by RP-HPLC on a microbore column. Fractions were collected as absorbance peaks, divided in two, and subjected to FGF-1 and Syn-1 immunoblot analysis and protein sequencing. *D,* samples of purified human recombinant FGF-1, rat recombinant Syn-1, and murine recombinant S100A13 were initially resolved by RP-HPLC to identify the retention time for each protein (data not shown) and then resolved as a mixture by RP-HPLC.

absorbance feature of the FGF-1 and p40 Syn-1 aggregate (Fig. 1*B*) may be due in part to the extinction coefficient of S100A13 (Fig. 1, *C* and *D*). Since it was possible to resolve a single fraction from RP-HPLC whose retention time was altered by treatment with temperature and the chaotropic agent, guanidine HCl, we suggest that S100A13 is a component of a multiprotein FGF-1- and p40 Syn-1-containing aggregate and that S100A13 is also present in this aggregate as a protein with a blocked amino terminus.

It is important to note that the major absorption peak in Fig. 1*C,* resulting from the denaturation of the fraction (Fig. 1*B*) containing FGF-1, p40 Syn-1, and S100A13, exhibited a distinct retention time, yet immunoblot analysis of this major absorption peak demonstrated the presence of low levels of FGF-1 and p40 Syn-1 (data not shown). Since members of the S100 gene family are known to self-associate (21) and to associate with membrane phospholipids (22), it is possible that the major absorption peak in Fig. 1*B* may contain S100A13 aggregates as well as other peptidic and non-protein components such as acidic phospholipids. Thus, denaturation of the multimeric aggregate in Fig. 1*B* containing p40 Syn-1, S100A13, and FGF-1, with temperature and treatment with guanidine HCl resulted in only a partial disruption of the aggregate. In addition, we cannot eliminate the possibility that the FGF-1, p40 Syn-1, and S100A13-containing peak resolved in Fig. 1, *A* and *B,* is the result of nonspecific protein aggregation under RP-HPLC conditions. However, it is noteworthy that recombinant S100A13 elutes from immobilized heparin between 0.2 and 0.4 M NaCl (data not shown), and recombinant p40 Syn-1 elutes from heparin-Sepharose at 0.7 M NaCl (5). Since the brain-derived, post-heparin-Sepharose fraction containing p40 Syn-1, FGF-1, and S100A13 was isolated as a high affinity (1.5 M NaCl elution peak) heparin-binding fraction prior to resolution by RP-HPLC, it is likely that S100A13 and p40 Syn-1 gained high heparin binding affinity through their ability to associate with FGF-1 prior to analysis by RP-HPLC.

Interestingly, automated Edman degradation of the FGF-1 fraction (Fig. 1*C*) derived from treatment of the FGF-1, S100A13, and p40 Syn-1 aggregate (Fig. 1*B*) with temperature and guanidine HCl also failed to yield information. However, automated Edman degradation of LysC fragments derived from the FGF-1 peak (Fig. 1*C*) demonstrated that it was present as a protein with a blocked amino terminus that has previously been characterized as FGF-1$\beta$ (residues 1–154) (23). Unfortunately, however, it was not possible to collect sufficient material from the p40 Syn-1 peak described in Fig. 1*C* for structural analysis, but it was possible to confirm the identity of this peak by Syn-1 immunoblot analysis (data not shown). Likewise, automated Edman degradation of the remaining peaks described in Fig. 1*C* did not yield any information, and sufficient material was not available for analysis by LysC digestion. These data imply that the ovine brain-derived, heparin-binding and denaturant-sensitive aggregate resolved by RP-HPLC (Fig. 1*B*) contains at least FGF-1$\beta$, p40 Syn-1, and S100A13. Furthermore, these data also provide an *in vivo* correlate to the presence of FGF-1 and p40 Syn-1 as a dena-

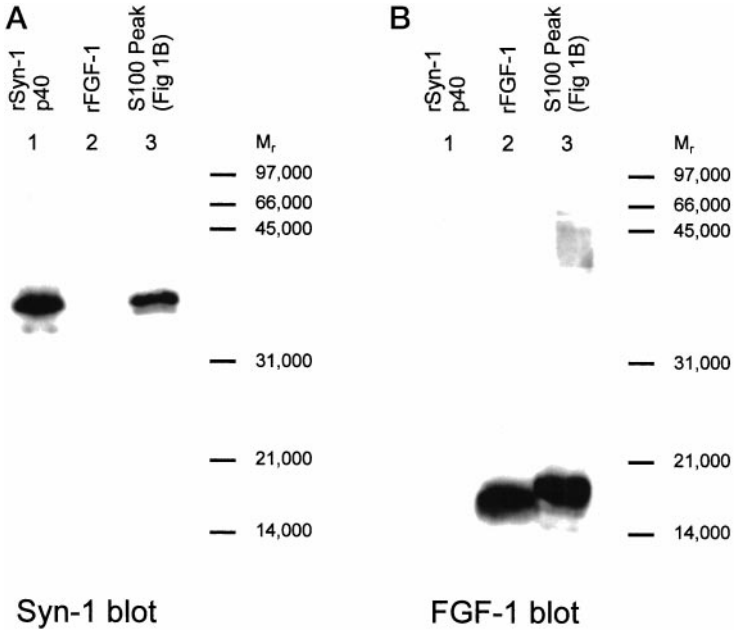


FIG. 2. **Immunoblot analysis of the S100-containing fraction.** The fraction corresponding to the major chromatographic peak shown in Fig. 1*B* was divided in two, and each aliquot was independently resolved by SDS-PAGE (12.5% acrylamide) under reducing conditions as described under "Experimental Procedures." *Lane 1* contains 50 ng of recombinant rat Syn-1 protein; *lane 2* contains 50 ng of recombinant human FGF-1$\alpha$ (residues 21–154); *lane 3* contains the chromatographic peak obtained from Fig. 1*B*. *A* represents the Syn-1 immunoblot and *B* the FGF-1 immunoblot. Note that the FGF-1 species present in the S100A13 peak (*lane 3*) migrates with a higher relative molecular weight than the recombinant species (FGF-1$\alpha$, residues 21–154), and this is consistent with its identification as the full-length form of FGF-1 (FGF-1$\beta$, residues 1–154) [18].

turant-sensitive aggregate that is released into the extracellular compartment in response to temperature stress *in vitro* (5).

*The S100A13-binding, Anti-allergic, and Anti-inflammatory Drug, Amlexanox, Represses the Stress-induced Release of FGF-1 and p40 Syn-1 in Vitro*—Because (i) S100A13 was purified as an aggregate with FGF-1 and p40 Syn-1 from ovine brain, (ii) elevated FGF-1 levels are associated with inflammatory environments *in vivo* (24), (iii) Syn-1 is released as a p40 fragment in response to temperature stress (5) and is required for heat shock-induced FGF-1 secretion (13), (iv) the anti-inflammatory and anti-allergic compound, amlexanox, binds S100A13 (Ref. 25; GenBank™ accession number AB001567), (v) amlexanox is able to interfere with the release of intracellular granules from basophils and mast cells (26), and (vi) NIH 3T3 cells express the S100A13 transcript (data not shown), we sought to determine whether amlexanox would be able to modify the stress-induced release of FGF-1 and p40 Syn-1 from NIH3T3 cells *in vitro*. NIH 3T3 cell FGF-1:$\beta$-galactosidase (Gal) and p65 Syn-1 co-transfectants were subjected to temperature stress (90 min, 41.5 °C) as described previously (13) in the absence and presence of amlexanox. The conditioned medium was treated with dithiothreitol, adsorbed to heparin-Sepharose, and the presence of FGF-1:$\beta$-gal and Syn-1 assessed by immunoblot analysis as described previously (2). As shown in Fig. 3, *A* and *B*, amlexanox was able to repress the release of both FGF-1:$\beta$-gal and p40 Syn-1 in response to temperature stress. The inhibition of FGF-1:$\beta$-gal and p40 Syn-1 release was dependent upon the concentration of amlexanox and was within the concentration range that exhibits pharmacologic effects as an anti-allergic and anti-inflammatory agent (27–29). Similar amlexanox concentrations were also able to inhibit the release of FGF-1 from FGF-1 NIH 3T3 cell transfectants *in vitro* (Fig. 5). Because of the possibility that amlexanox may also possess broad inhibitory activity upon conventional cellular secretion, we evaluated the ability of amlexanox to repress the release of a synthetic form of FGF-1 engineered to enter into the conventional ER-Golgi-mediated secretion pathway (30). Exposure of NIH 3T3 cells stably transfected with the FGF-4 signal peptide sequence: FGF-1 chimera (30) to amlexanox did not result in inhibition of the constitutive secretion of the FGF-1 chimera (data not shown). Furthermore, the forced secretion of FGF-1 is known to induce a prominent transformed NIH 3T3 cell phenotype *in vitro* (30), and amlexanox was unable to modify this phenotype. Additionally, the p40 extravesicular fragment of Syn-1 is constitutively released in p40 Syn-1 NIH 3T3 cell transfectants, and this release is potentiated by heat shock treatment (13). Whereas this constitutive release of p40 Syn-1 occurs by an unknown pathway, the temperature-enhanced release of p40 from these cells appears to utilize the regulated FGF-1 release mechanism (13). Thus, we examined the ability of amlexanox to influence the release of p40 Syn-1 from the NIH 3T3 cell p40 Syn-1 transfectants, and we observed that amlexanox was not able to block the constitutive release of p40 from cells maintained at 37 °C (Fig. 4, *lanes 1*, *3* and *4*). However, amlexanox was able to repress the temperature-sensitive component of p40 Syn-1 release (Fig. 4, *lane 5 versus lane 2*) *in vitro*. Finally, all experiments were monitored for cell lysis and changes in the permeability of the plasma membrane by measuring the levels of the cytosolic enzyme lactate dehydrogenase in the conditioned media of all samples, and no significant differences in extracellular lactate dehydrogenase levels were observed among the various treatments and conditions. We therefore suggest that amlexanox, the S100A13-binding compound, is able to significantly repress the temperature stress-induced release of both FGF-1 and p40 Syn-1.

*Chemical Modification of Amlexanox Reduces its Ability to Repress the Release of p40 Syn-1 and FGF-1 in Response to Temperature Stress in Vitro*—To provide a structural basis for the ability of amlexanox to repress the release of FGF-1 and p40 Syn-1 from its full-length (p65 Syn-1) precursor *in vitro*, we obtained several chemical derivatives of amlexanox (Fig. 5) with variable anti-allergic and anti-inflammatory activities. Each amlexanox derivative was evaluated for its ability to

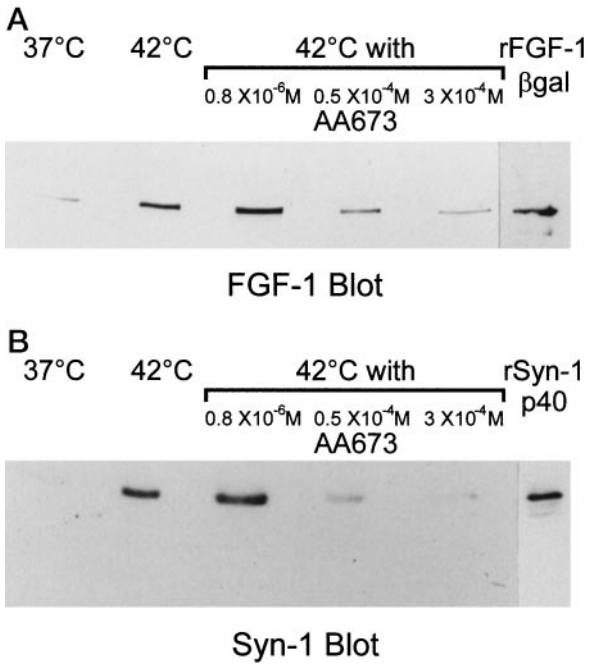


FIG. 3. **The effect of amlexanox on the release of the FGF-1:β-Gal chimera and p40 Syn-1 into media conditioned by NIH 3T3 cell FGF-1:β-Gal and p65 Syn-1 co-transfectants exposed to temperature stress.** Media were conditioned by two 150-mm dishes of NIH 3T3 cells stably transfected with human FGF-1:β-Gal and rat p65 Syn-1 by exposure to 41.5 °C for 90 min. The media were collected and resolved by 10% acrylamide SDS-PAGE under reducing conditions followed by immunoblot analysis for FGF-1 and Syn-1 as described under "Experimental Procedures." *A,* FGF-1 immunoblot analysis. *1st lane,* media derived from FGF-1:β-Gal and p65 Syn-1 NIH3T3 cell co-transfectants maintained at 37 °C for 90 min. *2nd lane,* media derived from FGF-1:β-Gal and p65 Syn-1 NIH3T3 cell co-transfectants following heat shock in the absence of any drug; *3rd* to *5th lanes,* media derived from FGF-1:β-Gal and p65 Syn-1 NIH3T3 cell co-transfectants following heat shock in the presence of $0.8 \times 10^{-6}$, $5 \times 10^{-5}$, and $10^{-4}$ M AA673 (amlexanox), respectively; *6th lane,* 50 ng of recombinant human FGF-1α:β-Gal and 50 ng of recombinant rat p40 Syn-1. *B,* Syn-1 immunoblot analysis; the filter was stripped according to the manufacturer's instructions (Amersham Pharmacia Biotech) and re-probed for Syn-1. Lane descriptors are identical to those described in *A*.

decrease the release of both FGF-1 and p40 Syn-1 from NIH 3T3 cell FGF-1 transfectants in response to temperature stress *in vitro*. As shown in Fig. 5, immunoblot analysis of media conditioned by heat shock in the presence or absence of either amlexanox (AA673) or one of its derivatives revealed that while derivatives AA617 and AA648 were able to repress FGF-1 release, derivative AA777 was not able to do so. Syn-1 immunoblot analysis of these samples also demonstrated that amlexanox derivatives AA617 and AA648 but not derivative AA777 were able to inhibit the release of p40 Syn-1 from endogenous p65 Syn-1 in response to temperature stress (data not shown). These data suggest that either substitution of the isopropyl side chain with an acetyl group or deletion of the amino group at the opposite end of the molecule does not reduce the ability of amlexanox to repress FGF-1 and p40 Syn-1 release in response to temperature stress *in vitro*. However, substitution of the isopropyl side chain with a methyl group generates an amlexanox derivative that is unable to repress the release of either FGF-1 (Fig. 5) or p40 Syn-1 (data not shown) in response to heat shock. These data suggest that the major functional group within the structure of amlexanox that is able to modify FGF-1 and p40 Syn-1 release is the hydrophobic side chain, including the maintenance of at least a two-carbon unit.

## DISCUSSION

We have utilized primarily *in vitro* methods to characterize the mechanism for the extracellular release of FGF-1 as a non-conventional release pathway since brefelden A, a drug which disrupts the classical ER-Golgi secretion pathway, does not inhibit the release of FGF-1 in response to temperature stress (2, 3). Within the extracellular compartment *in vitro*, FGF-1 exists as a latent Cys-30 homodimer (3) associated with the p40 extravesicular domain of Syn-1 (5). This aggregate is detergent- and reducing agent-sensitive, and electrophoretic mobility shift analysis anticipated the presence of additional components (5). In order to determine whether the information obtained from the characterization of the FGF-1 release pathway *in vitro* could be applied to an *in vivo* situation, we pursued the isolation of a multiprotein aggregate from ovine brain that contained FGF-1 and p40 Syn-1, and we report that the brain-derived FGF-1:Syn-1 aggregate also contains the calcium-binding protein, S100A13 (16), a member of the S100 gene family of EF-hand-containing $Ca^{2+}$-binding modulator proteins (17).

The S100 gene family members (31) encode low molecular weight acidic polypeptides containing two $Ca^{2+}$-binding EF-hand motifs flanked by two hydrophobic domains (17, 32). S100A13 also exhibits these structural features, and in contrast with other S100 gene family members, it contains a carboxyl-terminal domain that is rich in basic amino acid residues (16). Whereas all tissues express at least one member of the S100 gene family, the cellular distribution is specific for members of the S100 gene family (17). In this regard it is interesting that like FGF-1 (1) and Syn-1 (33), S100 proteins are highly enriched in neural tissue (34, 35). However, S100A13 expression is not limited to neural tissue, and with the possible exception of leukocytes, S100A13 expression is ubiquitous in other organs, tissues, and cell types (16) including its presence in NIH 3T3 cells. Interestingly, some members of the S100 gene family have been found to be associated with the nucleus (36), although most are cytosolic and can be associated with the endoplasmic reticulum and actin stress fibers (37). However, like FGF-1 (2), several S100 gene family members are released from cells *in vitro* (17, 38) despite the absence of a conventional signal peptide sequence for classical ER-Golgi-mediated secretion. Furthermore, even though no specific cell-surface receptor has been identified for these extracellular S100 gene family members (34, 39), some do possess extracellular functions including the regulation of neuronal differentiation (40–42), the stimulation of melanoma and glial cell proliferation and migration (35, 43), and chemotactic activities (44, 45). Indeed, it is interesting that FGF-1 is also able to accomplish these functions as well (1).

Since S100A8 and S100A9 are released from monocytes through a novel ER-Golgi-independent pathway that requires an intact tubulin network (38), it is not perhaps surprising that S100A13 may be a component of the FGF-1 and p40 Syn-1 ER-Golgi-independent release pathway. Indeed, the release of S100A8 by monocytes and activated neutrophils coincides with the release of the pro-inflammatory cytokine, interleukin (IL)-1α by these cells (46). This is interesting since the IL-1 and FGF gene family prototypes most likely evolved without classical signal peptide sequences from a common primordial gene (23). The IL-1 and the FGF gene family prototypes also exhibit sequence homologies (47, 48) including very similar crystallographic structures (49, 50). Like S100A8 and the IL-1 prototypes, FGF-1 is also expressed during inflammatory situations *in vivo* (24) and is thought to be released during these situations. Thus, it is also not surprising that the anti-allergic and anti-inflammatory S100A13-binding compound, amlexanox ((25) GenBank™ accession number AB001567), is able to re-

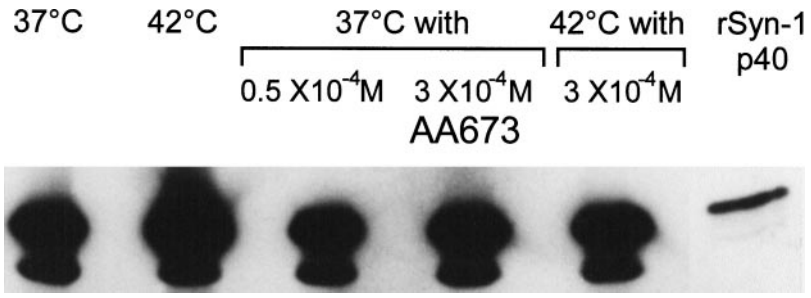


FIG. 4. **Amlexanox does not repress the release of constitutively secreted p40 Syn-1 from NIH 3T3 cell p40 Syn-1 and FGF-1:β-Gal co-transfectants.** Media were conditioned by two 150-nm dishes of NIH 3T3 cells stably co-transfected with FGF-1:β-Gal and rat p40 Syn-1 after exposure to temperature stress (41.5 °C, 90 min) with or without treatment. The media were collected and resolved by 10% acrylamide SDS-PAGE under reducing conditions followed by immunoblot analysis for Syn-1 as described under "Experimental Procedures." *1st lane,* media derived from NIH 3T3 cell FGF-1:β-Gal and p40 Syn-1 co-transfectants maintained at 37 °C for 90 min. *2nd lane,* media derived from NIH 3T3 cell FGF-1:β-Gal and p40 Syn-1 co-transfectants following heat shock. *3rd* and *4th lanes,* media derived from NIH 3T3 cell FGF-1:β-Gal and p40 Syn-1 co-transfectants maintained at 37 °C in the presence of $5 \times 10^{-5}$ and $3 \times 10^{-4}$ M AA673 (amlexanox), respectively. *5th lane,* media derived from NIH 3T3 cell FGF-1:β-Gal and p40 Syn-1 co-transfectants following heat shock in the presence of $3 \times 10^{-4}$ M, AA673. *6th lane,* 50 ng of recombinant rat p40 Syn-1.

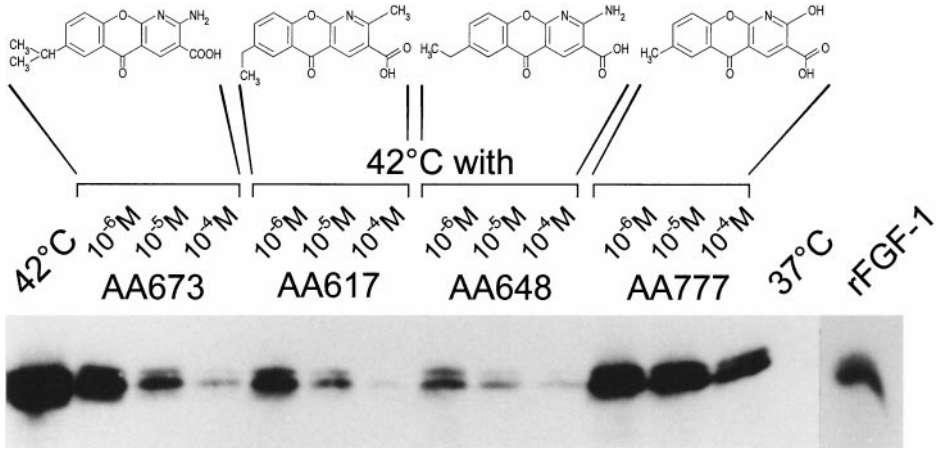


FIG. 5. **The effect of amlexanox and its derivatives on the release of FGF-1 into media conditioned by NIH 3T3 cell FGF-1 transfectants exposed to temperature stress.** Media were conditioned by three 150-mm dishes of NIH 3T3 cells stably transfected with FGF-1α (residues 21–154) after exposure to 41.5 °C for 90 min with or without amlexanox and its derivatives. The media were collected and resolved by 12.5% acrylamide SDS-PAGE under reducing conditions followed by immunoblot analysis for FGF-1 as described under "Experimental Procedures." *1st lane,* media derived from NIH 3T3 cell FGF-1 transfectants following heat shock in the absence of any drug; *2nd* to *4th lanes,* media derived from NIH 3T3 cell FGF-1 transfectant following heat shock in the presence of $1 \times 10^{-6}$, $1 \times 10^{-5}$, and $1 \times 10^{-4}$ M AA673 (amlexanox), respectively; *5th* to *7th lanes,* media derived from NIH 3T3 cell FGF-1 transfectants following heat shock in the presence of $1 \times 10^{-6}$, $1 \times 10^{-5}$, and $1 \times 10^{-4}$ M AA617, respectively; *8th* to *10th lanes*, media derived from NIH 3T3 cell FGF-1 transfectants following heat shock in the presence of $1 \times 10^{-6}$, $1 \times 10^{-5}$, and $1 \times 10^{-4}$ M AA648, respectively; *11th* to *13th lanes,* media derived from NIH 3T3 cell FGF-1 transfectants following heat shock in the presence of $1 \times 10^{-6}$, $1 \times 10^{-5}$, and $1 \times 10^{-4}$ M AA777, respectively; *14th lane*, media derived from NIH 3T3 cell FGF-1 transfectants maintained at 37 °C for 90 min; *15th lane*, 50 ng of recombinant human FGF-1α. The structure of amlexanox (AA673) and its derivatives are also shown.

press the release of FGF-1 and p40-Syn-1 in response to temperature stress *in vitro*.

Although we have not determined whether S100A13 is released in a brefelden A-insensitive manner with FGF-1 and p40 Syn-1 in response to cellular stress *in vitro*, our data do suggest that S100A13 may be at least a component of the intracellular multiprotein aggregate involved in the regulation FGF-1 release. Interestingly, S100 proteins (22) Syn-1 (8) and FGF-1 (4) are all well characterized as acidic phospholipid-binding proteins and since the p40 Syn-1 and FGF-1 components of the aggregate released from cells in response to heat shock are sensitive to denaturing agents, it is possible that the ability of these proteins to interact with phosphatidylserine may be a common feature of other unknown protein components of this release pathway. Indeed, gel shift analysis of the denaturant-sensitive component of the low heparin-binding affinity p40 Syn-1 and FGF-1 aggregate (5) anticipated the presence of a protein (~10 kDa) with an apparent molecular weight similar to that predicted from the S100A13 cDNA (16).

Since the FGF prototypes are well known for their angiogenic potential (47, 48) and tumor metastasis and growth is dependent upon tumor angiogenesis *in vivo* (51, 52), it is also not surprising that the expression of many of the S100 gene family members correlates with metastatic tumor potential (53–56), and in one instance is diagnostic for human melanoma metastatic potential (57, 58). Indeed, it is possible that S100 gene family members support neoplastic and pro-inflammatory situations *in vivo* by their ability to participate in the release pathway for extracellular signal peptide-less angiogenic and inflammatory signals such as FGF-1 and IL-1α. Interestingly, several S100 gene family members are also known to interact with members of the annexin gene family (59–61), and like Syn-1 and S100 gene family members, the annexins are $Ca^{2+}$-dependent acidic phospholipid-binding proteins (62). Annexin II is a particularly interesting member of this family since it has been implicated in the regulation of exocytosis (63, 64), and this activity appears to be mediated by its ability to be associated with S100A10 (p11) to form an annexin $II_2$-$p11_2$ aggregate (65, 66). Whereas annexin II is known to interact with plasminogen and plasminogen activators on the cell surface (67), annexin II, like S100A13 and FGF-1, does not contain a classical signal sequence for ER-Golgi-mediated secretion (63, 68).

Although it is not known whether S100A13 is able to interact with annexin II on the inner surface of the plasma membrane, annexins I through V were identified together with S100A13 as amlexanox-binding proteins (25).

The ability of amlexanox to repress the release of FGF-1 and the extravesicular p40 domain of p65 Syn-1 from NIH 3T3 cells in response to heat shock is noteworthy since S100A13 is an amlexanox-binding protein (25). Thus it is possible that amlexanox may interfere with the putative interaction between intracellular S100A13 and the FGF-1 and Syn-1 aggregate. Since amlexanox does not repress the appearance of the constitutively released p40 Syn-1 fragment *in vitro*, it is possible that amlexanox may be able to target the interaction between S100A13 and the putative FGF-1 and p65 Syn-1 aggregate. However, it is also possible that amlexanox may be interactive with other intracellular acidic phospholipid-binding proteins including members of the annexin gene family. Indeed, it is intriguing that the S100A13 (22), FGF-1 (4), and Syn-1 (8) components of this novel release pathway bind membrane phospholipids, since the ability of amlexanox to interfere with either the metabolism or the membrane release of lipid mediators of the inflammatory response, such as arachidonic acid derivatives, has served as the basis to explain the mechanism of action of amlexanox pharmacology (26–29).

Access to structural analogs of amlexanox has enabled us to define the importance of the isopropyl side chain as a functional element of its ability to repress the heat shock-induced release of FGF-1 and p40 Syn-1 *in vitro*. Since the modification of the amino group as well as conversion of the isopropyl side chain to an ethyl group did not influence the effectiveness of amlexanox to repress FGF-1 release in response to temperature stress, we suggest that these derivatives may serve as appropriate negative controls for these *in vitro* studies. Indeed, the general effect of these drugs on membrane permeability was measured by the release of lactate dehydrogenase, and amlexanox was not able to augment the presence of lactate dehydrogenase in the extracellular compartment under any condition. Interestingly, cromolyn, the parent drug used as the model for the development of amlexanox (69, 70), was also able to inhibit in a dose-dependent manner the release of FGF-1 and p40 Syn-1 from NIH 3T3 cell FGF-1 transfectants in response to temperature stress, but the data are not shown because contrary to amlexanox, cromolyn treatment resulted in significant dose-dependent increases in membrane permeability as measured by the presence of cytosolic lactate dehydrogenase in the extracellular compartment.

These data also reinforce the biological significance of the FGF-1 release pathway previously elucidated by the use of *in vitro* methods (2–5, 13) since insight into the role of S100A13 as a potential participant in the FGF-1 release pathway was derived from *in vivo* data using extracts of ovine brain. Since (i) the brain-derived aggregate appears to be partially resistant to both temperature denaturation and treatment with guanidine HCl, (ii) the known components of the brain-derived multiprotein aggregate bind acidic phospholipids, and (iii) the functional group within the structure of amlexanox responsible for the inhibition of FGF-1 and p40 Syn-1 release *in vitro* is the non-polar isopropyl side chain, we anticipate that phospholipid metabolism may not only play an important role in the mechanism of FGF-1 homodimer release but also in the regulation of the unknown intracellular responsibilities assumed by the FGF-1 monomer. With regard to the latter, we suggest that since S100 gene family members are well characterized as calcium-binding proteins that have calmodulin-like activities (17, 32, 35, 45), this function may involve structural aspects of the filamentous cytoskeleton and targets of the $Ca^{2+}$/calmodulin-dependent protein kinases.

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